Universidade do Minho
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Audience effects in *Oreochromis mossambicus* males on a territorial intrusion context

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UMinho | 2014

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Dissertação de Mestrado
Mestrado em Ecologia

Trabalho efetuado sob a orientação do
Professor Doutor Rui Oliveira
e supervisão da
Professora Doutora Fernanda Cássio

outubro de 2014

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Título dissertação □/tese ■

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Ano de conclusão: 2014

Designação do Mestrado ou do Ramo de Conhecimento do Doutoramento:

Mestrado em Ecologia

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Universidade do Minho, 31/10/2014

Assinatura: António João Almeida Roleira

Acknowledgments

The accomplishment of this thesis would not have been possible without the support of several people, to whom I would like to express my sincere gratitude.

Special thanks go to my supervisor Rui Oliveira, for his permanent availability and guidance, which were fundamental to the accomplishment and success of this thesis. I am also very grateful for the possibility of doing this research while ensuring the management of ISPA-IU fish facility.

I would also like to express my gratitude to the Minho University advisor Fernanda Cássio that since the first contact gave full support to my application and project. Without this availability and help in the connection with the university this thesis would not have been possible.

A special acknowledgment goes to Olinda Almeida for all the ideas and experience shared, and most important the friendship and availability. From you I learned the great experimental potential of tilapia, the basis of their welfare, and how to identify their behavioural activity.

I also want to thank Ana Félix and Gonçalo Oliveira for the support in laboratorial and statistical analyses respectively. Their precious help gave a great contribute to this work.

A word of recognition to Diana Rodrigues for the scientific drawing of tilapia used in this thesis, and for all helpful comments.

I am also grateful to my colleagues, namely Ana Cruz, Ana Faria, Ana Faustino, Ana Lopes, André Monteiro, João Lopes, José Pestana, Júlia Pinho, Leonor Galhardo, Magda Teles, Paulo Frias, Pedro Coelho, Pedro Vieira, Natália Madeira, Raquel Martins, Rodrigo Abreu and Sara Cardoso. I thank the sharing of scientific thoughts and experiences, as well as the help on taking care of the fish facility.

Last but not least, I am deeply grateful to my family for their unconditional love, support and tireless encouragement.

Financial support from Fundação para a Ciência e a Tecnologia (EXCL/BIA-ANM/0549/2012) is also gratefully acknowledged.

Audience effects in *Oreochromis mossambicus* males on a territorial intrusion context

Abstract

In the natural environment animal communication occurs on a network with several potential signallers and receivers. Given that in a signalling interaction sender and receiver can be observed by a third-party who will collect relevant information, it would be advantageous for those who interact to modulate their behaviour according to the presence of an audience (audience effect). In this study we investigated the audience effect in males of the African cichlid *Oreochromis mossambicus*. Specifically, we investigated the influence of two factors: *i)* exposure to an audience (presence/absence); and *ii)* exposure to an intruder (presence/absence), on the behaviour and steroid hormone levels. We found an audience effect in males' behaviour resulting in an increase of attention directed towards the audience when intruder is absent, which reveals an interest of the focal male in the audience, and an increase of the agonistic behaviour towards intruders in the presence of the audience. This increase is particularly noticeable in the escalation index, in the frequency of bites and displays. The presence of an audience also had an effect on steroid hormone levels. Cortisol levels were higher in the treatment where the focal male had an audience present but the intruder was absent. Conversely, testosterone levels were higher when the intruder was present and audience was absent. We found positive between interacting behaviours directed to audience and the hormones concentration of 11-ketotestosterone and cortisol. We also found negative correlations between agonistic behaviours and testosterone concentration. Together these results indicate an audience effect in the response to a territorial intrusion, which is inversely related to androgen levels.

Audience effects in *Oreochromis mossambicus* males on a territorial intrusion context

Resumo

No ambiente natural a comunicação animal ocorre numa rede com vários potenciais emissores e recetores. Dado que a interação de sinais entre emissor e recetor pode ser observada por terceiros que se podem apropriar de informação relevante, é vantajoso para os intervenientes modular o seu comportamento de acordo com a presença de uma audiência (efeito de audiência). Na presente tese investigámos o efeito de audiência em machos do ciclídeo africano *Oreochromis mossambicus*. Em particular, investigámos a influência de dois fatores: *i)* a exposição a uma audiência (presença/ausência); e *ii)* a exposição a um intruso (presença/ausência), no comportamento e nos níveis hormonais. Foi encontrado um efeito de audiência no comportamento dos machos, resultando no aumento da atenção dirigida à audiência quando o macho estava ausente, o que revela um interesse do macho focal na audiência, e um aumento dos comportamentos agonísticos dirigidos aos intrusos quando a audiência estava presente. Este efeito é particularmente visível no índice de escalada, na frequência de mordidas e de exibições. A presença de uma audiência teve também um efeito nos níveis de hormonas esteroides. Os níveis de cortisol foram mais elevados no tratamento em que o macho focal estava em presença da audiência e com o intruso ausente. Por outro lado, os níveis de testosterona foram mais elevados com o intruso presente e a audiência ausente. Foi detetada uma correlação positiva entre comportamentos de interação dirigidos à audiência e a concentração das hormonas 11-ketotestosterona e cortisol. Foi também detetada uma correlação negativa entre os comportamentos agonísticos e a concentração de testosterona. Em conjunto, estes resultados indicam um efeito de audiência na resposta a uma intrusão territorial, que é inversamente relacionada com os níveis de androgénios.

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1. Background

1.1. Social plasticity and Darwinian fitness

In the natural environment animals need to adapt their social behaviour to daily changes in the social environment and to transitions between life-history stages, and the ability to do so (called social plasticity) affects their Darwinian fitness (Oliveira, 2012). Therefore, social plasticity is a social competence that provides the capability of an individual to adjust its social behaviour depending on obtainable social information. To understand this social competence an integrative approach should be used to realise how different cues lead to different changes in animals' brains and how it translates into different behavioural responses (Taborsky and Oliveira, 2012).

1.2. Social competence and the use of public information

Social competence can be evaluated by the way animals use information that is available in their social group. According to Dall et al. (2005), the informed an individual, the better it can adjust its behaviour to the demands of a changeable environment. This information can have two different sources, as it can be acquired personally or socially. As Dall et al. (2005) describes, direct interactions with the environment produce personal information, that frequently do not correspond directly to the behaviour of the information collector.

The socially acquired information can be obtained by observing the behaviour of other animals, which might respond actively to the behaviour of the receiver (Dall et al., 2005). This information is generated by the activities of animals' social neighbours (conspecific or not) and can be obtained by two distinct pathways: *i)* signals; and *ii)* cues (inadvertent social information) (Danchin et al., 2004). Signals correspond to intentional information emitted by animals, and this information could be reliable or could intend to deceive the receiver (Dall et al., 2005). The inadvertent social information comprises "social cues" which transmit discrete information, usually about the spatial location or presence or absence of features, and "public information", which transmits graded information about the quality of features (Dall et al., 2005; Danchin et al., 2004). The emitter of these cues has no intention to transmit information, it just emerges of its actions to local observers. Usually, the inadvertent social information is reliable for bystanders due to the fact that this information is not intentionally produced (Danchin et al., 2004).

The use of public information, in particular, can be found in several cases on nature, for example, in breeding habitat selection (Doligez et al., 2003, 2002), in learning of local habitat depletion (Dall et al., 2005), and generally to assess the quality of environmental resources (Valone and Templeton, 2002; van Bergen et al., 2004).

All the ways of acquisition of social information previously mentioned (signals, social cues and public information) favour social learning. Valone & Templeton (2002), referring to previous works, concluded that the social learning of new skills has been well documented in a large variety of species in both the field and laboratory, implying a widespread social phenomenon. Also Galef & Laland (2005) found an empirical evidence of social influence on food choice, tool use, patterns of movement, predator avoidance, mate choice and courtship in several species. The signals used in animal communication can reach relatively large distances and therefore encompass several potential signallers and receivers. This observation suggests that the evolution of communication behaviour happens in a context of communication networks instead of communication dyads (Oliveira et al., 1998).

1.3. Communication networks

According to McGregor (2005), "*a communication network is a group of several animals within signalling and receiving range of each other*". In fact, communication cannot occur in isolation, as it is an inherently social behaviour. One of the many examples of this communication network is *Betta splendens* male behaviour that: *i)* increase (on the presence of a female observer) the intensity of conspicuous displays that can be used in communication with both males and females; and *ii)* decrease in highly aggressive displays that are solely directed to males. This suggests that male fights may provide both signals and cues not only to the direct opponent, but also to the viewers of the interaction (Doutrelant et al., 2001). Another example of this communication network is the general tendency of an individual to copy flee-responses of an entire flock (or herd) as response to fright or stress signs of other animals that enhances the use of social cues (Danchin et al., 2004).

In most cases, this socially acquired information is assumed as beneficial. However Giraldeau et al. (2002) advocated that situations happen when the use of this social information could bring potential disadvantages. For example, the authors refer that different situations where the consequences of incompatibility between personally acquired information and socially acquired

information, and the consequences of informational cascades (that correspond to situations giving rise to behavioural copying based on very little information) imply possible drawbacks in acquiring social information.

To understand this communication network is necessary to turn the attention to different participants, namely the contestants and the bystanders (Earley and Dugatkin, 2002). If the study is based on bystanders and how they extract information from signalling interaction between others, we are examining eavesdropping effects. When the focus of the study is the process of how individuals interact in the presence of a third party, we are analysing audience effects, as detailed below.

1.3.1. Eavesdropping

Eavesdropping is the ability of gathering information on the quality and motivation of territorial neighbours (Naguib et al., 2004). This phenomenon has been observed in a wide range of animals such as fishes (Earley and Dugatkin, 2002,Oliveira and McGregor, 1998), birds (Peake et al., 2001) and primates (Anderson et al., 2013). Some authors like Peake (2005) divide this behaviour into two main types: *i)* interceptive eavesdropping, when predators locate preys by extracting locating prey cues from prey signals; and *ii)* social eavesdropping, which consists on extracting information from a neighbour signalling interaction.

Concerning eavesdropping, Oliveira et al. (1998) confirmed that *B. splendens* monitor aggressive interactions between conspecific males and use that information in subsequent agonist interactions with those males. Johnstone (2001) demonstrated that "*eavesdropping is favoured whenever the costs of losing an escalated fight exceeds the value of the contested resource, and that its equilibrium frequency is greatest when costs are relatively high*". Other studies point out that some capabilities could be enhanced by an eavesdrop effect like the ability of some fish to make transitive inferences in the social rank of neighbours by observation only (Grosenick et al., 2007).

The eavesdropping has also been assessed in a interspecific view, for example in the work of Anderson et al. (2013), where image scoring for potential cooperativeness was measured in a non-human primate *Cebus apella*. In this work, the tufted capuchin monkeys show to be able to socially evaluate humans after observing third-party interactions that involve a helpful intervention or the absence of help.

1.3.2. Audience effects

Similarly to eavesdropping, audience effects also imply a third-party situation where individuals that are present, but do not take part in, signalling interactions between others are defined as audiences. In this section we focus on the individual signaller of a communication network, and how the bystanders influence the signaller behaviour. Matos & Schlupp (2005) define audience effects as changes in the signalling behaviour of one individual (during an interaction between individuals) caused by the presence of conspecifics.

The main challenge to the signaller is to compete (or cooperate) with other signallers and deal with the presence of several receivers. For example, Plath et al. (2008) demonstrated that *Poecilia mexicana* male alters is matting preference when in a presence of a conspecific male audience in order not to reveal its true preference. Another evidence are the pilferage avoidance strategies used by *Sciurus carolinensis* in the presence of a conspecific audience (Leaver et al., 2007). As an example of a cooperative signalling, we can mention the role of synchronized calling in anti-bat-predator behaviour of a tree frog, where Tuttle & Ryan (1982) verified that bat *Trachops cirrhosus* is less likely to respond to synchronous calls than to asynchronous calls proving the benefits of this cooperative behaviour.

In order to deal with the presence of several receivers, signallers need to solve two main problems which are: *i)* how to "connect" with a specific target; and *ii)* how to communicate in the presence of other bystanders besides the main target. An example of solving the first problem is the matched counter-singing found in *Melospiza melodia* (Beecher et al., 1996), where male have about nine distinct song types and share some of these song types with their neighbours. When neighbours counter-sing they use identical sound types proving a capability to communicate with a specific target. In what refers to communication in the presence of bystanders, Matos & McGregor (2002) demonstrate the changes induced by different conspecific sex audiences in *B. splendens* males. They found that when a male audience is present subjects tried significantly more bites and take less time near the opponent than with a female audience.

These audiences could be categorised into two different types: *i)* evolutionary audiences; and *ii)* apparent audiences (Matos and Schlupp, 2005). By evolutionary audiences they define individuals that were historically common in the environment of the signaller and that may have induced selection on the form and content of signalling behaviour as the example of the bird song that is

widely accepted to have a dual function, both as a signal to attract females and in male-male competition.

Apparent audiences are defined by individuals that affect the behaviour of the signaller only when they are present and detected, meaning that the effects produced by this type of audience are triggered only when the audience is present (Matos and Schlupp, 2005). According to this definition, most of the abovementioned studies are examples of apparent audiences.

Bearing in mind the above, it is concluded that the terms "audience" and "audience effects" have been used in different contexts over different studies. In some studies, like in Evans & Marler (1994), audience is referred to as the direct receiver of the signal although it triggers a change in behaviour. Nonetheless, in Matos and Schlupp (2005), the audience is not the main receiver of the signals, but acts as a potential non-targeted receiver.

There are several studies in audience effects conducted with very different groups of animals, including mammals (Kurzban et al., 2007; Vázquez et al., 2012), amphibians (Tuttle and Ryan, 1982), birds (Beecher et al., 1996; Naguib et al., 2004), fishes (Doutrelant et al., 2001; Oliveira et al., 1998) and insects (Fitzsimmons and Bertram, 2013). In the next paragraphs, some of the most relevant audience effects studies will be detailed.

1.3.2.1. Audience effects and behaviour

This is clearly one of the most explored aspects of audience effects. From the existing studies, we will explore the ones that provide an overview of those effects. This is the case of the work of Doutrelant et al. (2001), where the authors investigated if the presence of a conspecific audience leverages intra-sexual aggressive behaviour in Siamese fighting fish (*B. splendens*) males. They established that in the presence of a female audience, males increased the intensity of conspicuous displays that are used in communication with both males and females, and decreased highly aggressive displays that are only directed towards males.

Another study states that these audience effects in *B. splendens* are context dependent (Dzieweczynski et al., 2005). In this study, the influence of two contexts (audience of different sexes and presence or absence of a nest) on the aggressive behaviour of Siamese fighting fish males was investigated. The study confirmed the influence of the sex of the audience, as well as the impact of the territorial status of the focal males on their aggressive behaviour. It also revealed a solid relationship between audience effects and the existence of nest.

Dzieweczynski & Perazio (2012) explored another condition that influences audience effects on a male-male interaction in the Siamese fighting fish: familiarity. In this study, fighting fish males were first presented to an individual that became later an audience. After that, the focal males interacted with each other in the presence of three possible audiences: *i)* the first presented individual (i.e., familiar audience); *ii)* a new unfamiliar audience; or *iii)* without audience. The authors found that audience familiarity influenced opponent-directed gill flaring and tail beats, even if they were affected differently by the sex of the audience that was present.

One example of audience effects on behavioural studies made in mammals is an investigation made by Dibitetti (2005). The author accessed audience effects on the food-associated calls in *Cebus apella nigritu*s. In this study, the author found an effect of the audience on the latency to give food-associated calls. Specifically the time elapsed until the food finder gave the first food-associated call decreased with the presence and density of nearby individuals and increased with the distance from other individuals. It was also found that the latency to call was longer towards females than towards males. The author concluded that the audience effect and the effect of the sex of the finder in *C. a. nigritus* allows to conclude that they use these vocalizations deceptively by withholding information about the presence of a food source.

Although audience effects reported so far refer to relations with conspecifics, audience effects have also been observed at an inter-specific level. One example of an inter-specific audience effect is Pinto et al.(2011). The authors explored the effect of an audience on cooperative cleaning of cleaner wrasses *Labroides dimidiatus*. These cleaners learn to feed against their preferences if that allows them to access additional clients, meaning cooperatively eating ectoparasites instead of cheating by eating client mucus under natural conditions. In this study the authors found evidence that cleaners instantly increased their levels of cooperation in the presence of a potential bystander client. It was also established that bystanders react with avoidance to any event of cleaners cheating on their current client. Bearing in mind the above, Pinto et al. (2011) validated that image scoring by an audience leads to increased levels of cooperation in a non-human animal.

1.3.2.2. Audience effects and hormones

Typically androgens are thought of as the sex steroids that control male reproduction. Yet, recent evidence showed that androgens can also be affected by interactions between conspecifics (Oliveira et al., 2002). Bearing this in mind, it is relevant to evaluate how interactions within a communication network affect steroid levels. There are two different possible effects of

communication networks on hormone levels: *i)* audience effect – where the interveners are accessed; and *ii)* eavesdropping effect – in which the effects on the bystanders are the point of interest.

Dzieweczynski et al. (2006) assessed the audience effect in 11-ketotestosterone (hereinafter referred to as "11-KT") levels on *B. splendens* males. In their work, the authors investigated if 11-KT levels were influenced by nesting status and presence of an audience. Considering the results, they suggested that 11-KT levels were influenced by reproductive status and audience presence and were most likely modulated by territorial behaviour and social environment. A more recent work by Dzieweczynski & Buckman (2013) relates an acute exposure to 17α-ethinylestradiol to a disruption of audience effects on a male-male interaction in *B. splendens*, concluding that this acute exposure decreases the ability of males to communicate with multiple individuals simultaneously. This is particularly significant because, in that case, endocrine disruptor exposure could change population structure, since selection would favour individuals that are able to quickly adapt their signalling behaviour as a function of social context. In a different work, Hirschenhauser et al. (2013) investigated the link between the winner (and loser) effect with testosterone levels and with the audience effect in future interactions, and concluded that male Japanese quails' post-conflict testosterone were linked to the audiences, meaning that the androgen concentration was more related with the observers' perception of the challenge rather than to winning or losing a fight.

Regarding bystander effects, Oliveira et al. (2001) found that in *O. mossambicus*, watching fights raised hormone levels in bystanders, since 30 minutes after observing fights among conspecifics, the concentrations of testosterone and 11-KT were significantly elevated in the urine of the bystander, with 11-KT remaining above basal concentrations for a further 3 hours after the fight. In opposition, in the control situation they verified a continuous decreasing in both steroids, explained by the normal daily variation in basal urinary androgens, being highest in the early morning, and declining during the day.

1.4. *Oreochromis mossambicus* as a model

Oreochromis mossambicus (Peters 1852), (Figure 1), is a mouthbrooder cichlid fish that belongs to the tilapiine cichlid tribe, endemic of Africa and Middle East. The tilapiine tribe includes three main genera which differ in their mating and parental behaviour: *i)* Tilapia (40 species) which are

substrate spawners, monogamous, and with biparental care; *ii)* Sarotherodon (30 species) which are monogamous, with biparental or paternal mouthbrooding; and *iii)* Oreochromis (10 species) which are polygamous and maternal mouthbooders (Trewavas, 1982).

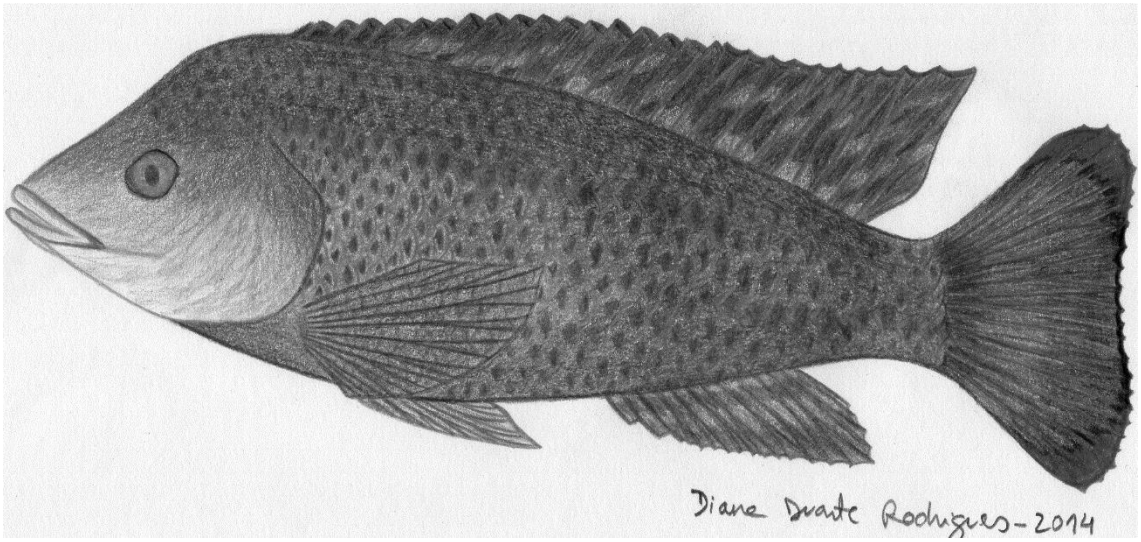

Figure 1: Illustration of *Oreochromis mossambicus* (Peters 1852) male kindly provided by Rodrigues, D.

As other species of the tilapiine group, since the 1930s *O. mossambicus* has been dispersed worldwide for different reasons, such as biological control of insects and aquatic weeds, as bait, as a food source and for aquaria. As an exotic species, it became the most widely distributed group worldwide (Canonico et al., 2005).

Because of this worldwide distribution and variety of uses, it became important to extend the knowledge of this group. This led to a rich literature on this species that provides a great background for further research. This factor, in association with the fact that our laboratory have an extended experience with this model, provided the basis for the selection of this species as our experimental model for the present study.

Previous work on *O. mossambicus* in our lab focused on the dynamics of social relationships and on the role of hormones on the establishment of dominance hierarchies (Oliveira and Almada, 1998, 1996a, 1996b), and on the biology of welfare for the improvement of housing and handling (Galhardo et al. (2012, 2011, 2009).

As models of hormonal studies the research of Martins et al. (2009) and (Oliveira et al., 2005, 2002, 2001) embody straight approaches to take into account in our work.

1.5. Aim of the dissertation

The aim of this study is to assess if the presence of an audience and/or an intruder induces modifications in the behaviour and hormone levels of *O. mossambicus* males. The results of this study will contribute to a better understanding of the mechanisms underlying audience effects and will further clarify the use of contextual information by fish in social decision making.

2. Materials and methods

2.1. Animals and housing

The 48 *O. mossambicus* males (mean ± standard deviation: length, 10.5 ± 0.65 cm; body mass, 36.18 ± 6.67 g) used in this study were obtained from a stock maintained at the ISPA-IU fish facility (Lisbon, Portugal). All focal animals used were dominant males (characterized according to Oliveira (1995)) housed in stock aquaria of 160 L with fine gravel substrate, a double filtering system (both sand and external biofilter, TETRA) and constant aeration (Figure 2). Each stock aquarium had fourteen animals (eight males and six females) at 26±2 °C, with a photoperiod of 12h light: 12h dark. Fish were daily fed *ad libitum* with commercial cichlid sticks (TETRA). Water quality was monitored once a week with Pallintest kit® for nitrites (0.2 - 0.5 ppm), ammonia (<0.5 ppm) and pH (7.2 – 7.4).

Figure 2: General view of the stock aquarium.

2.2. Experimental setup

Focal males were placed individually in the experimental tanks (30 L tanks being all the remaining conditions the same as described earlier for the stock) 48 h prior to the test for acclimatization. During the acclimatization phase, all focal males established territories, built nests and adopted the nuptial coloration. In this acclimatization phase each experimental tank had visual access to a female audience tank (placed in 2/3 of the lateral side of focal tank (Figure 3)). That visual access was provided to simulate stock conditions by ensuring visual access to an audience in the

adaptation period for leveling cortisol to stock concentrations. We based this option on a study of Galhardo et al. (2011b) that concludes that males present lower cortisol levels when in stable social groups than when in social isolation.

In the inside border of the focal tank we placed an intruder tank with visual and chemical communication with the focal tank, but without access to the audience tank (neither visual nor chemical). An unidirectional mirror was positioned between focal and audience tanks to allow focal males to see the audience, but preventing the audience to see and interact with the focal males.

Females were chosen as an audience in order to promote a more affiliative and less competitive social context, considering that, in a previous study isolated males expressed an high motivation, comparable to the motivation for obtaining food, to come in close contact with females (Galhardo et al., 2011a). We chose unfamiliar females as an audience in order to prevent any effects of previous experience.

Four treatments were conducted: *i)* Treatment Intruder Audience (T.I.A.) – focal male in an intrusion context with visual contact with a female audience; *ii)* Treatment Intruder No-Audience (T.I.NA.) – focal male in an intrusion context without visual contact with female audience; *iii)* Treatment No-Intruder Audience (T.NI.A.) – focal male only in visual contact with female audience, but in the absence of intruder male; and *iv)* Treatment No-Intruder No-Audience (T.NI.NA.) – focal male in an isolation context (i.e. no audience and no intruder present).

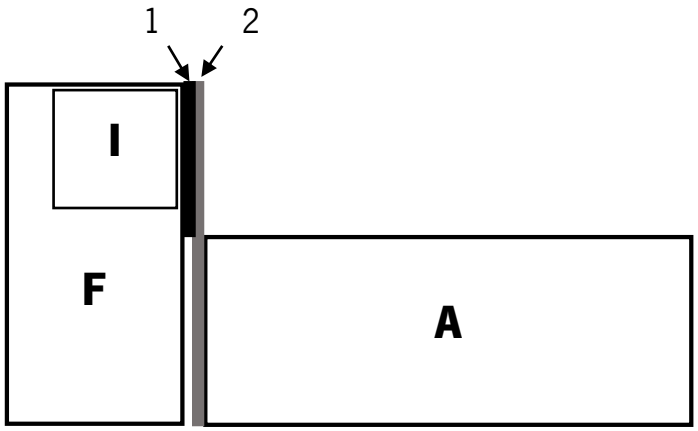

Figure 3: Top view of the experimental set up: F – Focal tank (glass); I – Intruder tank (glass; placed inside de focal tank) with holes to allow chemical communication; A – Audience tank (glass); 1 – Permanent barrier to prevent visual communication between intruders and audience; 2 – Unidirectional mirror to prevent audience to see and interact with focal males.

2.3. Sampling procedures

On the experimental day, five minutes before the trial (which started between 8:30 a.m. and 9:30 a.m.) a video camera was placed at the opposite side of the audience tank, allowing the visualization of the three tanks. In the groups without audience, immediately after starting the camera an opaque partition was placed between the one-way mirror and the audience aquarium. In all intruders groups, the intruder male was placed in the intruder tank two to three minutes after turning on the camera. The experimental treatment order was balanced between males.

2.3.1. Behavioural sampling

For behavioural quantification 30 minutes of video recording were analysed with Noldus The Observer XT 7.0 software (Noldus Information Technology). The behavioural patterns were characterized according to the patterns described in Table 1.

Table 1: Brief description of behavioural patterns.

Behavioural pattern*	**Description**
Frontal and lateral display	Behaviour towards an opponent in which the male remains motionless in the water column with fully erects dorsal and anal fins, with spread caudal and pelvics, and eventually branchiostegal membrane erected. If in parallel/antiparallel position to opponent, we considered lateral display, if facing the opponent and usually with mouth opened, we considered frontal display.
Biting	Fish approaches or attacks opponent with mouth opened and rams it in any part of the body, but especially in the flanks and near the head. Despite the existence of a glass, this behaviour is very obvious.
Pit Digging (Nest digging)	In vertical position or at an angle of 45°, with mouth opened, the male digs a depression on the substrate. Two main movements are included: with head downwards, mouth is pushed against substrate, filled with particles, which are ejected in the pit periphery and, in deeper pits, fish may swim up the slope pushing the substrate to the periphery.
Nipping substrate	With head downwards, body at an angle of 45°, and fins half erected, fish thoroughly nips out substrate; sand and particles may be carried to some distance before being expelled, while fish describes an apparent wandering route.

Table 1: Brief description of behavioural patterns (continued).

Behavioural pattern*	Description
Nipping at the surface	The fish, while swimming or floating near the surface, turns the head directly upwards and make nipping movements with the jaws.
Inactivity	Fish remains immobile in touch with the substrate or/and hovering in non-territorial areas.
Swimming	Natural fish displacement around the tank.
Glass interaction	Attempts to interact with the audience swimming pushed up against the glass, without attempt to attack.
Stationary position (floating)	Fish keeps a constant position in the water column. The body is in a horizontal position, dorsalis, analis and pelvics closed against the body, caudalis closed and tail beets. Pectorals movements as in a swimming backwards makes the fish remain in the same position.

*Adapted from Baerends and Baerends-Van Roon (1950), Oliveira (1995) and Galhardo et al. (2008).

2.3.2. Hormonal sampling

Immediately after the behavioural trial, the focal animal was quickly anesthetised (MS-222, Pharmaq; 300ppm) and blood samples were taken from the caudal vein (using 1ml syringes with 25 G/ 16mm needles). Blood sampling always took less than 4 minutes since the induction of anaesthesia to avoid effects of handling stress on sampled cortisol levels (Foo and Lam, 1993). Blood samples were centrifuged at 3000G for 10 minutes (temperature 4 °C) and the plasma was stored at −20 °C until all samples were collected.

Free hormone fraction was extracted from the plasma by adding diethyl ether to the sample to act as the steroid solvent (#1.00921.1000, Merck), and stirred (Janke & Kunkel, Ika-Vibrax-Vxr) for 20 minutes. The next step was to centrifuge samples (5 minutes, 1000 rpm, 4 °C, Heraeus, Megafuge 1.0R) and froze them (10/15 minutes, −80 °C) to separate the ether fraction (this process was repeated twice). The steroids were isolated by evaporating the ether in Speed-Vac (Savant instruments) for 20 minutes. Dried organic phase containing free steroid fraction was reconstituted with 1 ml buffer solution supplied with the Enzyme immunoassay (EIA) kit. Samples were stored at −20 °C until their analysis.

Steroid concentrations were measured by radioimmunoassay (RIA). For the cortisol RIA we used the commercial cortisol antibody from Fitzgerald (cat #20-CR-50, Rabbit anti-cortisol) raised in rabbit using cortisol-3-CMO-BSA as the immunogen. The cross reactivity of this antibody is 100% for cortisol, 36.0% for prednisolone, 5.7% for 11-desoxycortisol, 3.3% for corticosterone and < 0.7% cortisone. The marked hormone for cortisol was the [1, 2, 6, 7-3H] Cortisol (Amersham Biosciences, ref. TRK407-250uCi).

For the testosterone RIA we used the commercial testosterone antibody from RDI (Research Diagnostics Inc, cat #WLI-T3003, Rabbit anti-testosterone) with the following cross reactivity: 5α-Dihydrotestosterone 63%; s-1-testosterone 46%; 5α-Androstan-3α,17β-diol 4.1%; s-5-Androsten-3β,17β-diol 14%; 5α-Androstan-3,17-dione < 4.0%; epi-testosterone < 0.7%; Aldosterone < 4.0%; Hydrocortisone, < 0.02%, Progesterone < 0.2%; Estradiol < 0.5%; Dehydroepiandrosterone < 0.4%; Androstenedione 3.5%; Danazol < 2.0%. The marked hormone for testosterone was the [1, 2, 6, 7-3H] Testosterone (Amersham Biosciences, ref. TRK402-250mCi).

For the 11-KT RIA we used a custom made antibody for 11-KT kindly donated by D.E. Kime (specificity table for this antibody has been published in Kime and Manning, 1982). The titrated 11-KT was produced in-house from marked cortisol. The marked hormone for 11-KT was kindly donated by Comparative Molecular Endocrinology Group (Algarve University).

Intra-assay variability were 0.75% for cortisol; 2.68% for testosterone and 3.74% for KT.

2.4. Data analysis

All statistical analyses were completed using the statistical software Statistica V.12.0® (StatSoft Inc, USA, 1984–2013). An outliers analysis was performed and extreme values (i.e. > 3SD) were removed from the subsequent analysis.

We performed Kolmogorov–Smirnov and Shapiro–Wilk normality tests and operated a logarithmic transformation to the data that did not had a normal distribution (according to Zar, (2010)). Nevertheless, some of the variables analysed did not presented a normal distribution even after data transformation, and were still used in the ANOVA without transformation (i.e., we only used the logarithmic transformation of cortisol). This decision was based in Lindman, (1974) that states that the F-statistic is remarkably robust to deviations of normality and heterogeneity of variances and could still be used due to the lack of equivalent non-parametric tests.

Bearing in mind the above, a factorial ANOVA was used followed by planned comparisons of Least Squares (LS) means for behavioural and hormonal analysis among treatments T.I.A, T.I.NA., T.NI.A. and T.NI.NA. previously referred.

We conducted the behavioural analysis with two different approaches, as an audience communication approach and an intruder communication approach. The analyses were conducted on the frequency and duration of these interactions.

Referring to the audience communication approach, the only measure that gave us data about this approach was the glass interaction. Relating to the glass interaction, we run a factorial ANOVA considering as categorical predictors the presence (or absence) of an audience and the presence (or absence) of an intruder, and as dependent variables (one at a time): *i)* the frequency of glass interactions; and *ii)* the duration of glass interactions.

Concerning to intruder communication approach, we just found agonistic interactions. This analysis was conducted on all the groups included in the experiment, based on the frequency and duration of the most relevant agonistic interactions identified in the video analysis. Regarding the frequency of events, we ran a factorial ANOVA considering as categorical predictors the presence (or absence) of an audience and the presence (or absence) of an intruder, and as dependent variables (one at a time): *i)* the frequency of bites; *ii)* the frequency of displays; *iii)* the aggression (total frequency of aggressive events computed as the sum of the frequency of bites with the frequency of displays); and *iv)* the escalation index (calculated as the ratio between the frequency of bites and the aggression frequency).

In what concerns to the duration of events, we also ran a factorial ANOVA with the same categorical predictors. In this case, we considered as dependent variables the displays duration.

Regarding the hormonal analysis, we ran a factorial ANOVA to cortisol, 11-KT and testosterone concentrations with the abovementioned categorical predictors.

Since the main aim of our analysis is to understand the effect of an audience on an intrusion context, we performed the following planned comparisons of LS means: *i)* T.I.A. vs T.I.NA; *ii)* T.NI.A vs T.NI.NA; *iii)* T.I.NA. vs T.NI.NA; and *iv)* T.I.A vs T.NI.A..

Correlation analysis between behavioural and hormonal data has been performed with correlation matrices.

A significance value of $P \leq 0.05$ was used in all statistical tests.

2.5. Ethical note

The experiments described here were conducted in accordance with Portuguese legal standards on protection of animals used for experimental purposes and are part of a project with a permit (Ref. 0421/000/000/2013) from The Portuguese Veterinary Authorities (Direcção Geral de Alimentação e Veterinária, Portugal).

3. Results

In this section the empirical results of the experiment are detailed in three subtopics: behavioural analysis, hormonal analysis and hormone-behaviour correlational analysis.

3.1. Behavioural analysis

In order to characterise the average behaviour of each one of the treatments included in our experiment, we began by inspecting the behavioural sequences on The Observer XT 7.0. The results are depicted in Figure 4. We verified that the agonistic behaviour occurred in treatments T.I.A. and T.I.NA. (i.e., where intruders were present) and that treatment T.NI.NA. had a lower variety of behaviours. In other hand, treatment T.NI.A presented the highest amount of time in non-agonistic behaviours.

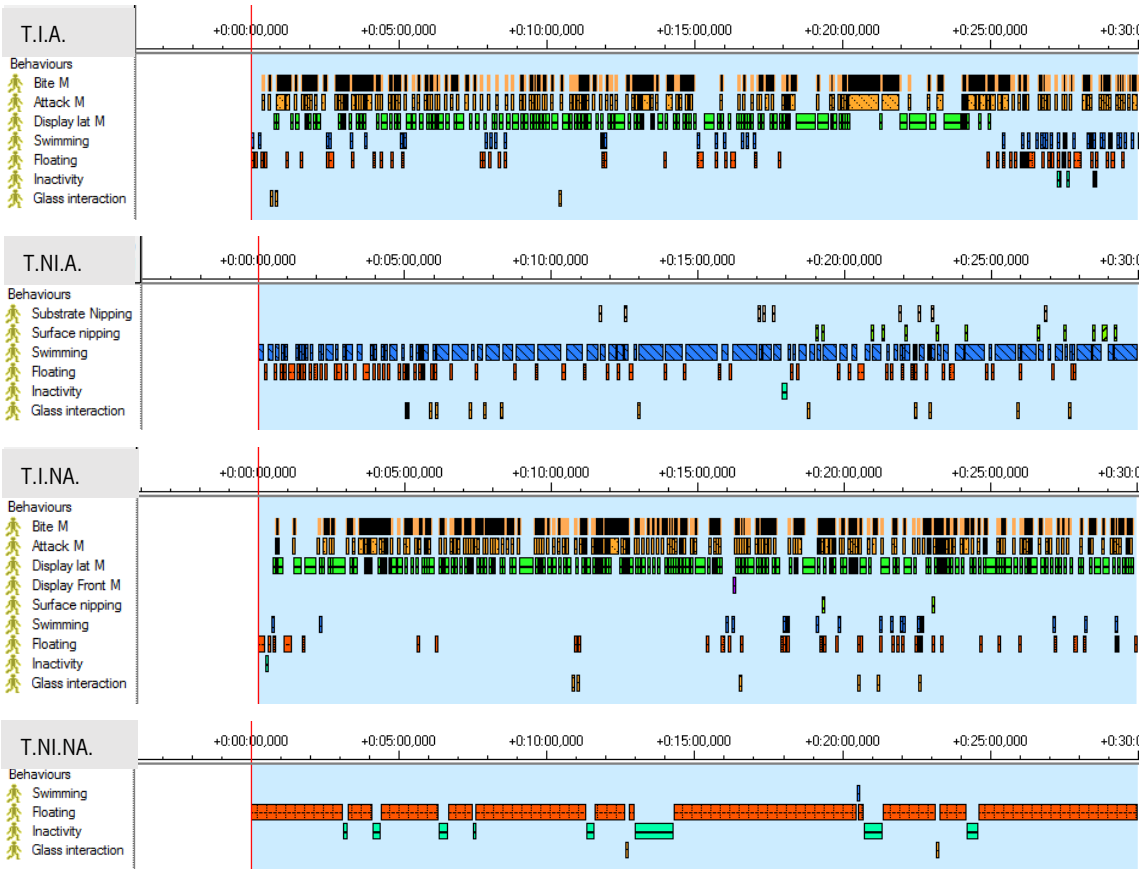

Figure 4: Behavioural visualisation of the four experimental groups in 30 minutes of interaction obtained with Observer XT 7.0; T.I.A. – Treatment Intruder Audience; T.NI.A. – Treatment No-Intruder Audience; T.I.NA. – Treatment Intruder No-Audience; T.NI.NA. – Treatment No-Intruder No-Audience.

Regarding the behavioural analysis the main results of our study (Table 2) are detailed below.

Table 2: Results of ANOVAs and Planed Comparisons on behavioural data. Bold highlighted values are significant at $P<0.05$; T.I.A. – Treatment Intruder Audience; T.I.NA. – Treatment Intruder No-Audience; T.NI.A. – Treatment No-Intruder Audience; T.NI.NA. – Treatment No-Intruder No-Audience.

Behaviours	**ANOVA**			**Planed comparisons of LS means**			
	Main effect of Audience (A)	**Main effect of Intrusion (I)**	**Interaction A x I**	**T.I.A. Vs T.I.NA.**	**T.NI.A. Vs T.NI.NA.**	**T.I.NA. Vs T.NI.NA.**	**T.I.A. Vs T.NI.A.**
Frequency of Glass Interaction	$F_{1,42}$=6.92 P=0.012	$F_{1,42}$=3.58 P=0.065	**$F_{1,42}$=9.55 P=0.004**	$F_{1,42}$=0.10 P=0.753	**$F_{1,42}$=17.18 P=0.0002**	$F_{1,42}$=0.75 P=0.391	**$F_{1,42}$=11.85 P=0.001**
Duration of Glass Interaction	$F_{1,42}$=3.00 P=0.091	$F_{1,42}$=3.01 P=0.090	**$F_{1,42}$=5.07 P=0.030**	$F_{1,42}$=0.13 P=0.721	**$F_{1,42}$=8.33 P=0.006**	$F_{1,42}$=0.14 P=0.710	**$F_{1,42}$=7.58 P=0.008**
Bite frequency	$F_{1,42}$=3.11 P=0.085	**$F_{1,42}$=62.04 P=0.000**	$F_{1,42}$=3.11 P=0.085	**$F_{1,42}$=5.93 P= 0.019**	$F_{1,42}$=0.00 P=1.000	**$F_{1,42}$=19.62 P=0.0001**	**$F_{1,42}$=44.36 P=0.000**
Display Frequency	$F_{1,42}$=3.55 P=0.066	**$F_{1,42}$=73.82 P=0.000**	$F_{1,42}$=3.55 P=0.066	**$F_{1,42}$=6.79 P= 0.013**	$F_{1,42}$=0.00 P=1.000	**$F_{1,42}$=23.61 P=0.00001**	**$F_{1,42}$=52.39 P=0.000**
Escalation index	$F_{1,42}$=3.91 P=0.054	**$F_{1,42}$=221.8 P=0.000**	$F_{1,42}$=3.91 P=0.054	**$F_{1,42}$=7.47 P= 0.009**	$F_{1,42}$=0.00 P=1.000	**$F_{1,42}$=87.51 P=0.000**	**$F_{1,42}$=135.8 P=0.000**
Aggression	$F_{1,42}$=3.31 P=0.076	**$F_{1,42}$=67.04 P=0.000**	$F_{1,42}$=3.31 P=0.076	**$F_{1,42}$=6.33 P= 0.016**	$F_{1,42}$=0.00 P=1.000	**$F_{1,42}$=21.28 P=0.00004**	**$F_{1,42}$=47.80 P=0.000**
Display Duration	$F_{1,42}$=0.18 P=0.677	**$F_{1,42}$=80.85 P=0.000**	$F_{1,42}$=0.18 P =0.677	$F_{1,42}$=0.32 P=0.572	$F_{1,42}$=0.00 P=1.000	**$F_{1,42}$=38.61 P=0.000**	**$F_{1,42}$=42.18 P=0.000**

Concerning interaction behaviours with the audience we found a significant interaction between independent variables audience and intruder, both in frequency and duration of glass interactions (Table 2).

When we depicted this analysis in the planned comparisons of LS means with the different treatments we found several effects. Regarding the frequency of interactions we found a significant difference when we compared treatments T.NI.A. and T.NI.NA (Table 2). We also found an effect

when we compared treatments T.I.A. and T.NI.A. (Table 2). In the analysis of the duration of glass interactions we found an effect when we compared treatments T.NI.A. and T.NI.NA and an effect when we compared treatments T.I.A. and T.NI.A. (Table 2). From these analyses and inspecting Figure 5, we conclude that the treatment T.NI.A (intruder absent and audience present) had a significantly higher frequency and duration of glass interactions than all other treatments.

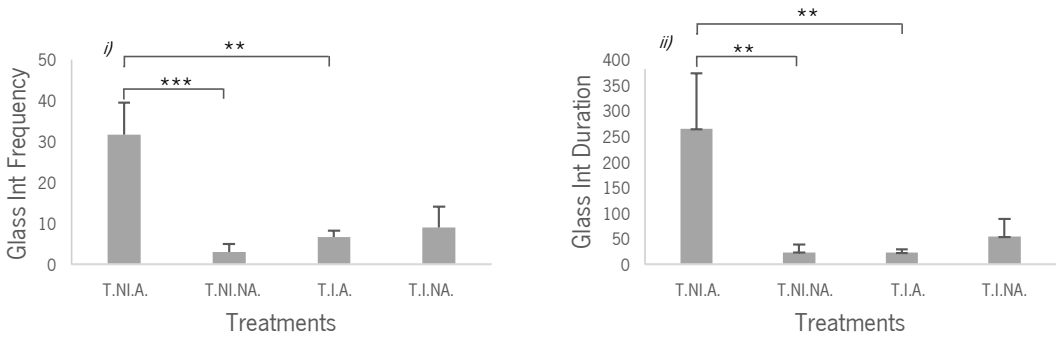

Figure 5: *i)* Mean values and Standard Error of the Mean (SEM) of frequency of glass interactions in treatments T.NI.A., T.NI.NA., T.I.A., T.I.NA.; *ii)* Mean values and SEM of duration of glass interactions in treatments T.NI.A., T.NI.NA., T.I.A., T.I.NA.; T.NI.A. – Treatment No-Intruder Audience; T.NI.NA. – Treatment No-Intruder No-Audience; T.I.A. – Treatment Intruder Audience; T.I.NA. – Treatment Intruder No-Audience; Asterisks indicate significant differences: $^{**}P < 0.01$; $^{***}P < 0.001$.

In what concerns to the frequency of agonistic events, (Table 2) we found a main effect of intrusion variable in all behaviours analysed. The planned comparisons of LS means between T.I.A. and T.I.NA revealed significant results on the: *i)* escalation index; *ii)* frequency of bites; *iii)* frequency of displays; and *iv)* aggression frequency (Table 2). In all the cases treatment T.I.A. presents a higher frequency of agonistic events (Figure 6).

We also found a significant effect when treatments T.I.NA. and T.NI.NA. were compared (Table 2). In all the behaviours analysed treatment T.I.NA. presented a higher frequency of agonistic events (Figure 6).

Significant effects were also found when treatments T.I.A. and T.NI.A were compared (Table 2). In all behaviours analysed treatment T.I.A. presented a higher frequency of agonistic events (Figure 6).

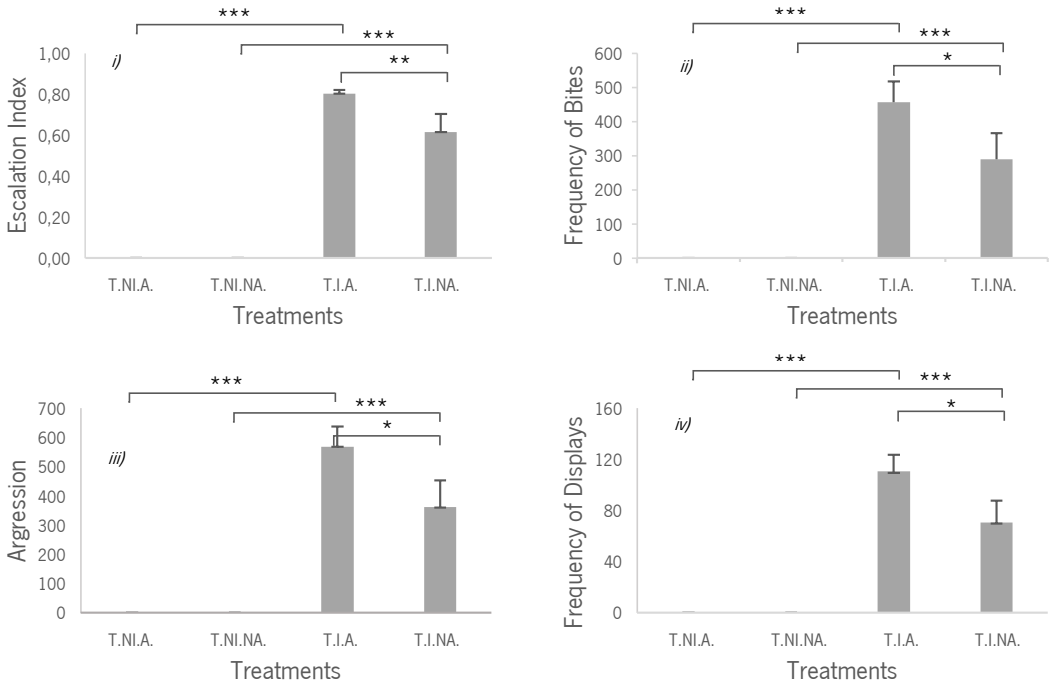

Figure 6: *i)* Mean values and SEM of the escalation index in treatments T.NI.A., T.NI.NA., T.I.A., T.I.NA.; *ii)* Mean values and SEM of frequency of bites in treatments T.NI.A., T.NI.NA., T.I.A., T.I.NA.; *iii)* Mean values and SEM of frequency of displays in treatments T.NI.A., T.NI.NA., T.I.A., T.I.NA.; *iv)* Mean values and SEM of aggressive events in treatments T.NI.A., T.NI.NA., T.I.A., T.I.NA.; T.NI.A. – Treatment No-Intruder Audience; T.NI.NA. – Treatment No-Intruder No-Audience; T.I.A. – Treatment Intruder Audience; T.I.NA. – Treatment Intruder No-Audience; Asterisks indicate significant differences: $*P < 0.05$; $**P < 0.01$;$***P < 0.0001$.

In the analysis of displays duration, we also found an main effect of the intrusion (Table 2). The planned comparison tests revealed significant effects when comparing treatments T.I.NA. and T.NI.NA., and when comparing treatments T.I.A. and T.NI.A (Table 2). In the analyses of the mean values of the treatments (Figure 7) we found that treatments with intruder absent never showed displays.

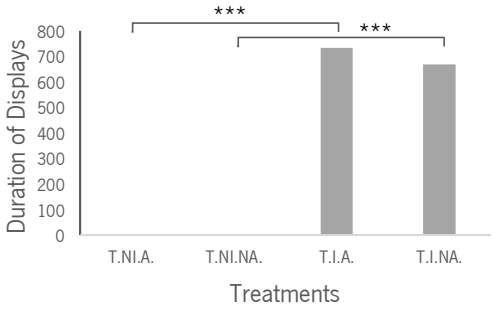

Figure 7: Mean values and SEM of the escalation index in treatments T.NI.A., T.NI.NA., T.I.A., T.I.NA.; T.NI.A. – Treatment No-Intruder Audience; T.NI.NA. – Treatment No-Intruder No-Audience; T.I.A. – Treatment Intruder Audience; T.I.NA. – Treatment Intruder No-Audience; Asterisks indicate significant differences:$***P < 0.0001$.

3.2. Hormonal analysis

Concerning the hormonal analysis the main results of our study are presented in Table 3.

Table 3: Results of ANOVAs and Planed Comparisons on hormonal data. Bold highlighted values are significant at $P < 0.05$; Underlined values represent a trend; T.I.A. – Treatment Intruder Audience; T.I.NA. – Treatment Intruder No-Audience; T.NI.A. – Treatment No-Intruder Audience; T.NI.NA. – Treatment No-Intruder No-Audience

Dependent variables	**ANOVA**			**Planed comparisons of LS means**			
	Main effect of Audience (A)	**Main effect of Intrusion (I)**	**Interaction A x I**	**T.I.A. Vs T.I.NA.**	**T.NI.A. Vs T.NI.NA.**	**T.I.NA. Vs T.NI.NA.**	**T.I.A. Vs T.NI.A.**
Cortisol	**$F_{1,41}$=7.83 *P*=0.008**	$F_{1,41}$=2.50 *P*=0.121	$F_{1,41}$=0.22 *P*=0.638	$F_{1,41}$=2.53 *P*=0.119	**$F_{1,41}$=5.74 *P*=0.021**	$F_{1,41}$=0.63 *P*=0.432	$F_{1,41}$=2.06 *P*=0.159
Testosterone	**$F_{1,41}$=10.43 *P*=0.002**	**$F_{1,41}$=4.40 *P*=0.042**	$F_{1,41}$=0.38 *P*=0.534	**$F_{1,41}$=6.94 *P*=0.012**	$\underline{F_{1,41}=3.65}$ $\underline{P=0.063}$	$F_{1,41}$=1.12 *P*=0.296	$\underline{F_{1,41}=3.60}$ $\underline{P=0.065}$
11-KT	$F_{1,41}$=0.80 *P*=0.376	$F_{1,41}$=1.68 *P*=0.202	$F_{1,41}$=1.94 *P*=0.171	$F_{1,41}$=2.45 *P*=0.125	$F_{1,41}$=0.13 *P*=0.718	$F_{1,41}$=0.01 *P*=0.945	$\underline{F_{1,41}=3.52}$ $\underline{P=0.068}$

Regarding the cortisol analysis (Figure 8), we found a main effect of the presence of an audience (Table 3). We also found an effect in the planned comparison between T.NI.A. and T.NI.NA. where cortisol concentration was higher in T.NI.A. (Table 3).

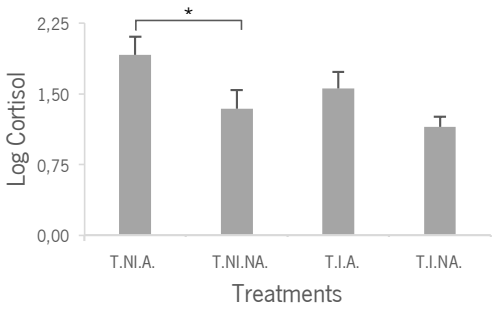

Figure 8: Mean values and SEM of cortisol in treatments T.NI.A., T.NI.NA., T.I.A., T.I.NA.; T.NI.A. – Treatment No-Intruder Audience; T.NI.NA. – Treatment No-Intruder No-Audience; T.I.A. – Treatment Intruder Audience; T.I.NA. – Treatment Intruder No-Audience; Asterisks indicate significant differences: $*P < 0.05$.

In what refers the testosterone analysis (Figure 9), we found a main effect of both audience and intruder variables (Table 3).

We also found an effect in the panned comparisons between treatments T.I.A. and T.I.NA., with higher testosterone concentrations in T.I.NA. (Table 3). Some other comparisons also revealed a trend, namely the comparisons between treatments T.NI.A. and T.NI.NA., with higher testosterone concentration in the T.NI.A. treatment and between treatments T.I.A. and T.NI.A., with higher testosterone concentration in the T.NI.A treatment (Table 3).

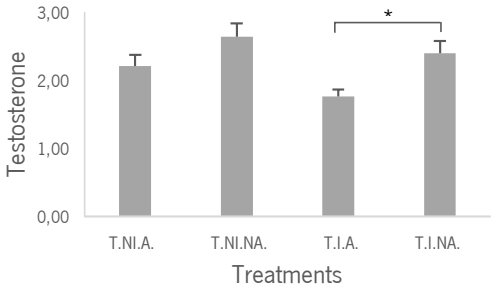

Figure 9: Mean values of the variation of testosterone in treatments T.NI.A., T.NI.NA., T.I.A., T.I.NA.; T.NI.A. – Treatment No-Intruder Audience; T.NI.NA. – Treatment No-Intruder No-Audience; T.I.A. – Treatment Intruder Audience; T.I.NA. – Treatment Intruder No-Audience; Asterisks indicate significant differences: $*P < 0.05$.

Finally, in what regards to 11-KT we could observe from Table 3 that there were no significant main effects. Analysing the mean values of the 11-KT (Figure 10), it could be expected some significant differences between T.I.A and other treatments, but when we deepened the contrast analysis, we found just a trend between treatments T.I.A. and T.NI.A that was not statistically significant (Table 3).

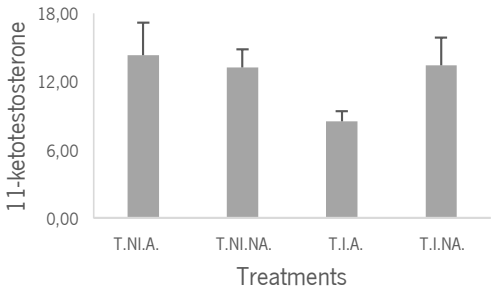

Figure 10: Mean values of the variation of 11-KT in treatments T.NI.A., T.NI.NA., T.I.A., T.I.NA.; T.NI.A. – Treatment No-Intruder Audience; T.NI.NA. – Treatment No-Intruder No-Audience; T.I.A. – Treatment Intruder Audience; T.I.NA. – Treatment Intruder No-Audience;

3.3. Correlational analysis

In addition to the behavioural and hormonal analysis, we have also searched for the existence of correlations between the agonistic behaviours and steroid hormones (Table 4).

Table 4: Correlation matrix between agonistic behaviours and hormones; Bold marked correlations are significant at $P < 0.05$; Underlined marked correlations express a trend

Agonistic variables	**Hormonal variables**		
	11-KT	**Testosterone**	**Cortisol**
Frequency of Glass interaction	0.120 *P*=0.433	-0.054 *P*=0.726	**0.392** ***P*=0.008**
Duration of Glass interaction	**0.302** ***P*=0.044**	0.333 *P*=0.828	**0.341** ***P*=0.022**
Frequency of bites	-0.189 *P*=0.215	**-0.299** ***P*=0.046**	-0.179 *P*-0.238
Frequency of displays	-0.211 *P*=0.164	**-0.335** ***P*=0.024**	-0.139 *P*=0.362
Escalation index	-0.270 *P*=0.073	**-0.358** ***P*=0.016**	-0.206 *P*=0.175
Aggression	-0.195 *P*=0.200	**-0.308** ***P*=0.039**	-0.173 *P*=0.257
Display duration	0.010 *P*=0.950	-0.097 *P*=0.526	-0.078 *P*=0.612

From the analysis of Table 4, we found positive correlations (highlighted in bold) between cortisol and the frequency and duration of glass interactions, and between 11-KT and the duration of glass interactions. We also found negative correlations (also highlighted in bold) between testosterone and several agonistic behaviours, namely the frequency of bites, the frequency of displays, the escalation index, attack time and aggression.

4. Discussion

In our study we found several behavioural and hormonal effects of audience and intruder presence in *O. mossambicus*.

Concerning the behavioural analysis, the first key finding is the main effect detected in the analysis of the glass interaction that gives us support to confirm that our focal males are aware of and have interest in the audience. In this analysis it is clear that in the treatment when focal males have no intruder and have an audience present (T.NI.A) they have significantly higher frequency of glass interactions and significantly longer glass interactions that reveal the male interest to engage in interactions with the females.

These outcomes are consistent with the results obtained by Galhardo et al. (2011a) where the authors measured *O. mossambicus* motivation to perform several tasks and found that males of this species are highly motivated to get access to a social partner.

In what relates to the agonistic interactions, the first highlighted result is the impact of intruder variation (presence/absence) in the aggressive behaviour of the focal male. In treatments where the intruder is absent (T.NI.A. and T.NI.NA.), the focal male does not express an aggressive behaviour (Figures 6 and 7).

When an intruder is present and audience varies (treatments T.I.A. and T.I.NA.), the most relevant results are achieved on the escalation index, with a higher ratio when audience is present. This index, together with the results of the frequency of other agonistic events, like the frequency of bites, frequency of displays and total frequency of aggressive events, which always presents a higher frequency of events in the presence of an audience, point towards a strong audience effect on the agonistic behaviour of *O. mossambicus* males. In particular, the results highlight that the presence of an audience enhances the frequency of aggressive events. The other effects found (in T.I.NA. Vs T.NI.NA. and T.I.A. Vs T.NI.A.) are related with the presence of the intruder that induces the agonistic response.

According to Doutrelant et al. (2001), *B. splendens* present a less aggressive behaviour in the presence of a female audience relatively to a male audience and to audience absence. In their study, the authors found no statistical difference between treatments without audience and with a male audience. However, they found a significant reduction of highly aggressive behaviours exclusively directed to males when a female audience was present and an increase of the displays

used in communication with both males and females. In our study, *O. mossambicus* revealed an opposite behaviour, namely a significant increase in the frequency and duration of direct agonistic behaviour to the intruder in the presence of an audience.

However, the differences between this species could be inherent to differences in paternal care behaviour of each specie. Different works in *B. splendens* (e.g., Dzieweczynski et al. (2005)), point out a reduction in aggression when both males have nests. This decrease of aggressive behaviour could be beneficial to do not risk destroying the nest with aggressive movements because in this specie the male provide parental care of the eggs. Yet, in *O. mossambicus* dominant males establish and defend their reproductive arenas only allowing females to get in when they are ready to mate (Oliveira, 1995) but they don't have paternal care. So, to *B. splendens* being less aggressive when a mate is close could denote a higher fitness and in *O. mossambicus* being more aggressive could also denote a higher fitness as it just means a better protection of the nest or of the territory(respectively), and therefore, explains different behavioural patterns.

From these results, it can be established that *O. mossambicus* present a modulation of the behaviour promoted by the presence of an audience. This modulation of behaviour in the presence of an audience with the purpose of enhances reproductive success has been reported in other species, such as in *P. mexicana* (Blum et al., 2008), *C. a. nigritus* (Dibitetti, 2005), and *T. guttata* (Dubois and Belzile, 2012), among others.

Several works also evaluated hormonal changes as a response to an audience effect (Dzieweczynski and Buckman, 2013; Dzieweczynski et al., 2006; Hirschenhauser et al., 2013). Given that in our study we find behavioural differences in agonistic behaviours, we decided to further investigate hormonal effects on cortisol, testosterone and 11-KT.

In what concerns to cortisol, we find a higher cortisol concentration in the treatment where they have an audience present but no intruder (T.NI.A.). According to the work of Galhardo et al. (2011b), males would present lower cortisol levels when they live in stable social groups, rather than when they are isolated. Considering this, we could expect a lower cortisol concentration when audience was present, and particularly when audience was present and the intruder was absent (T.NI.A.), since it would provide the possibility of territory establishment and female access.

Notwithstanding the above, the results obtained point in the opposite direction. One possible explanation for this result relates to our set-up design, particularly the existence of a unidirectional

mirror. This mirror was introduced to allow the focal male to see the audience in the adaptation period, and to prevent the audience to see and interact with the focal male, in order to make him focused in the intruder when intrusion occurs, since Doutrelant et al. (2001) also highlighted the importance of preventing focal-audience interactions. However, preventing audience feedback when no intruder is present, may have thwarted the social communication attempts (for example, courtship behaviours). As a consequence, the focal male increased the stress response. In the intruder groups, even if intruders are considered a threat, they provide behavioural (and presumably chemical) feedback to the focal fish agonistic actions. This answer can induce reduced levels of stress relatively to those situations where there is no response at all (which happens when audience is present without intruder present).

This interest in the audience feedback is supported by our data relatively to the abovementioned glass interaction. This interaction shows the importance to establish social communication for the focal male, since when audience is present and the intruder is absent (T.NI.A.) they spend significantly more time attempting to establish this communication.

Relating to testosterone, there is a main effect of the independent variables audience and intruder. We also find a significant effect when intruder is present. The treatment with absence of an audience (T.I.NA.) reveals a significant higher concentration of this hormone comparing with the audience treatment (T.I.A.). In addition, when the intruder is absent (T.NI.NA. and T.NI.A.) the testosterone concentration still tends to be higher in the audience absence treatment (T.NI.NA.). In treatments T.I.A. and T.NI.A. testosterone concentration tends to be higher in the intruder absence (T.NI.A.). Even if not statistically significant, this draws our attention to the fact that treatments where audience is present (T.I.A. and T.NI.A.) always exhibit lower testosterone concentration than those with audience absent (T.I.NA. and T.NI.NA.).

In other species testosterone is important to establish and maintain a high social status. For example, in a work of Earley and Hsu (2008) with *Kryptolebias marmoratus*, the authors concluded that animals with higher levels of testosterone are more aggressive and have lower latency to bite in social interactions. Therefore, and as all focal males establish a territory during the acclimatization period, we could expect that groups with audience would have a higher testosterone concentration. Also in the meta-analyses of the challenge hypothesis by Hirschenhauser and Oliveira (2006) the authors point out a relation between testosterone and aggressive behaviour in

fish. However, in this study the treatment with audience and intruder (T.I.A.) presence reveals the opposite.

Despite being unexpected, these results find support in the review of Oliveira and Oliveira (2014) that shows that although in most studies in humans, increased testosterone levels are associated with winnings, in some cases the opposite trend is observed.

Considering the ‘‘challenge hypothesis” (Wingfield et al., 1990) that predicts that seasonal patterns in circulating androgen concentrations vary as a function of mating system, male-male aggression and paternal care, Goymann (2009) proposed that to clarify the testosterone response in birds the different factors that may interfere in this hormone variation should be divided. Namely, he consider the seasonal testosterone response (R_{season}), the androgen responsiveness to male-male interactions ($R_{male\text{-}male}$), the androgen responsiveness of males to receptive females ($R_{male\text{-}female}$), the androgen responsiveness of non-social environmental cues ($R_{environment}$), and the potential androgen responsiveness ($R_{potential}$) to a better understanding of this hormone variation.

Concerning the 11-KT analysis, in the treatment where intruder and audience are present (T.I.A.) the 11- KT concentration presented a trend to be lower. This hormonal result is in line with the work in *B. splendens* by Dzieweczynski et al. (2006) where the authors concluded that 11-KT levels were lower in interacting males when a female audience was present. Nevertheless, this just occurs in hormonal analysis, since in the behavioural analysis (as detailed before) the agonistic response towards the intruder were enhanced in the presence of an audience.

Several authors (e.g., Dzieweczynski et al. (2006); Hirschenhauser et a., (2013)) found correlations between behaviour and hormones in audience effects studies. As it could embody important information we also performed those correlations.

Concerning to the correlation between the interaction behaviours with the audience and hormones, we find a positive correlation between 11-KT and the duration of the glass interaction, and also a positive correlation between cortisol and the frequency and the duration of the glass interaction.

The results of the correlation analysis highlight a negative correlation between testosterone and agonistic behaviours (frequency of bites, frequency of displays, escalation index and aggression). Even when we find a strong behavioural reaction (with a significant increase of agonistic behaviours in audience treatments previously referred) this is inversely reflected at physiological level.

The results of the androgen analysis find support in the work of Almeida et al. (2014a), that found that in *O. mossambicus* androgens are necessary for the expression of reproductive behaviours but not for the expression of aggressive behaviour, suggesting a mediator role in the former case and a moderator role in the later. The authors refer that the aggressive behaviour seems to be decoupled from androgens, being their action associated to the expression of reproductive behaviours. Considering that glass interaction is the most closed event related with sexual behaviour our results confirm this hypothesis.

The work of Oliveira et al. (1996) with *O. mossambicus* refers a strong correlation between the social status and androgens after group formation, pointing out that social interactions may have an important modulatory effect on sex steroid concentrations.

With a different approach, Rosvall et al. (2012), working with *Junco hyemalis* birds, suggest that behavioural variation in aggression may be more related to neural sensitivity to steroids and less related to circulating steroids.

5. Concluding remarks

In this study we extended the investigation of audience effects associated with the presence and absence of an intruder, both in terms of agonistic behaviour and hormonal response, to *O. mossambicus* males.

In particular, our work demonstrates the existence of an audience effect at behavioural level in *O. mossambicus* males that is characterized by an increase of the agonistic behaviour directed to the intruder when in the presence of a female audience.

Yet, as in *B. splendens* (Dzieweczynski et al., 2012, 2005), the audience effect could be context and familiarity depend in our model. Hence, future investigation could focus on verifying if the audience effect in *O. mossambicus* males is also context and familiarity dependent, and thus affected by: *i)* different audiences (such as male and mixed audiences); or *ii)* different familiarity relations, because in our work all animals are unfamiliar at the beginning of trail (i.e., focal male, intruder male and audience derive from different aquarium stocks).

We also found an audience effect at hormonal level with the increase of cortisol concentration when the focal male is in the presence of an audience (when the intruder is absent). These results could be influenced by the presence of a unidirectional mirror. For that reason, future work should be focused in exploring the effect of the presence of an unidirectional mirror in behaviour and hormones.

The results obtained also provide strong evidence of the existence, in *O. mossambicus,* of social plasticity (i.e., behavioural modulation ability in various social contexts). This capability is evidenced more precisely by modulating the agonistic behaviour as function of the presence of an audience. This could represent an adaptation to enhance their fitness performance when the audience is present, and, therefore, grant a higher social rank.

To better understand this plasticity ability it is crucial to explore how these behavioural changes are reflected in the brain. For that reason, we have also collected brain samples that will be the examined in a future work focused on the quantification of the differences, at brain level, of the immediate early genes (genes activated in rapid response to environmental changes) involved in the Brain Social Behaviour Network – BSBN (Taborsky and Oliveira, 2012). In fact, there is evidence that plasticity depends on the neurons' capability of showing short or long-lasting phenotypic changes in response to different stimuli and cellular scenarios (Pérez-Cadahía et al.,

2011). In order to do that, modifications in the immediate early genes evolved in this BSBN should be quantified, particularly in what concerns to *egr1* and *c-fos* genes.

In addition to the above, our investigation also contributed to understand how behaviour varies in the social network of this species. This is of particular relevance since the better tools we have to understand behaviour variation across social networks, the better we can understand how changes in the natural environment will affect social interactions and, as a result, quantify the threat level of climatic or human promoted changes to species living in the affected environments. Some work have been done with this theme in other species, namely related to environmental changes (Wong and Candolin, 2014) and concretely to pollution (Scott and Sloman, 2004; Ward et al., 2008).

Finally, this study, in addition to the existing studies on audience effects and on eavesdropping (McGregor, 2005) contributes to a better understanding of communication evolution as a network.

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